

Article

Solid Phase Assembly of Fully Protected Trinucleotide Building Blocks for Codon-Based Gene Synthesis

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Abstract: The use of pre-formed trinucleotides, representing codons of the 20 canonical amino acids, for oligonucleotide-directed mutagenesis offers the advantage of controlled randomization and generation of “smart libraries”. We here present a method for the preparation of fully protected trinucleotides on solid phase. The key issue of our strategy is the linkage of the starting nucleoside to the solid support via a traceless disulfide linker. Upon trinucleotide assembly, the disulfide bridge is cleaved under reducing conditions, and the fully protected trinucleotide is released with a terminal 3'-OH group.

Keywords: trinucleotide building block; gene library; protein engineering; codon; randomization

1. Introduction

A major goal of molecular biotechnology is the evolution/development of enzymes with desired and improved properties for industrial or pharmaceutical application [1,2]. Often the knowledge about structural details of the enzyme of interest is missing, such that rational design is not an option [2]. On the contrary, directed evolution offers the possibility, via random mutagenesis, of generating large protein libraries, which are screened for functionality, such that detailed structural information is not required [2]. Furthermore, combinatorial gene synthesis offers the option of restricting structural variations to residues of interest, i.e., those that are involved in a certain function such as catalysis. Nevertheless, there are a number of hurdles to be considered. For example, the size of gene libraries resulting from large random mutagenesis would be too big for practical screening [3]. Other challenges are the structure of the genetic code, where some amino acids are encoded by up to six triplets and others by just one, leading to a bias of the mutation, favoring those amino acids with multiple codons [4]. On the methodological side, directed evolution by error-prone PCR is a widely used strategy, which however, generates bias of mutation depending on the polymerase used [4]. Furthermore, the generation of stop codons cannot be prevented, which leads to abortion fragments instead of the full-length enzymes [5]. A possibility to overcome these problems is the use of trinucleotide building blocks (Figure 1), which represent codons of the canonical amino acids and thus permit the controlled synthesis of a fully or partially randomized gene library without stop codons or codon bias [6]. There are several but not easy ways to synthesize such trinucleotide building blocks, either in solution [7] or, as a more recent addition to the field, on soluble support [8,9]. We have previously developed a strategy for synthesis in solution of fully protected trinucleotide phosphoramidites [6] and, more recently, have succeeded in synthesizing trinucleotides on solid support. The preparation of

trinucleotides in solution can be carried out at a larger scale. However, it requires careful design of the strategy and orthogonal protection of individual groups. Thus, a pair of orthogonal protecting groups for the 5'- and 3'-OH functionalities needs to be designed, in order to avoid side reactions during trinucleotide synthesis. Stable protection of the phosphate moieties as well as of the heterocyclic bases throughout trinucleotide synthesis is essential. After each reaction step, isolation and purification of intermediate products is required, making trinucleotide assembly in solution a rather tedious and time-consuming procedure. Syntheses on support are advantageous in terms of efficiency. Products bound to the support and removal of excess reagents by simple washing facilitates the process and saves time. The future may lie in the usage of soluble polymers [8,9]. Soluble supports combine the advantages of solution chemistry and solid phase methods. However, they require a reaction scheme optimized for liquid phase assembly, which currently is still under investigation. Trinucleotide assembly on solid support can proceed by standard phosphoramidite chemistry and, thus, is a valuable strategy for the preparation of fully protected trinucleotides, until the major hurdles of supported oligonucleotide assembly on soluble polymers have been overcome. Our initial protocol for solid phase trinucleotide assembly has been described recently [10]. We here wish to report our studies regarding the choice of a suitable polymer, efficient conjugation chemistry for linking the starting nucleoside to the support, and detachment of the fully protected trinucleotide.

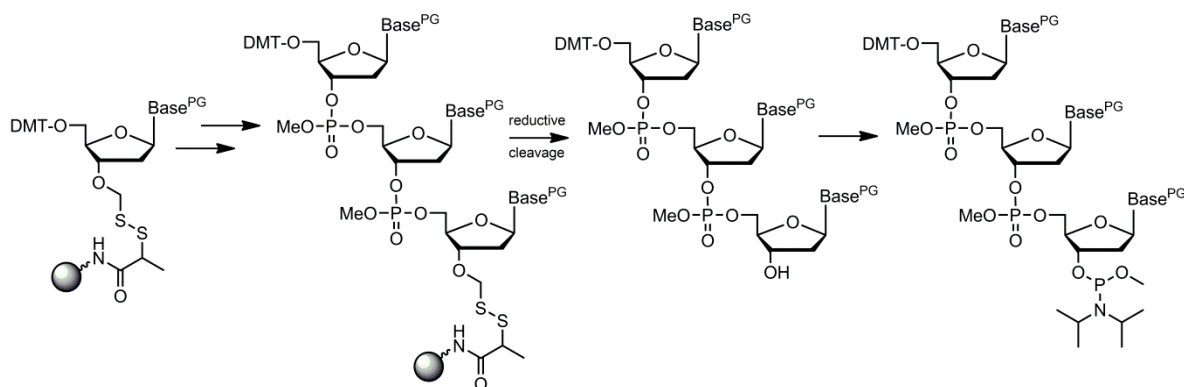


Figure 1. Reaction scheme for preparation of fully protected trinucleotide phosphoramidites.

2. Materials and Methods

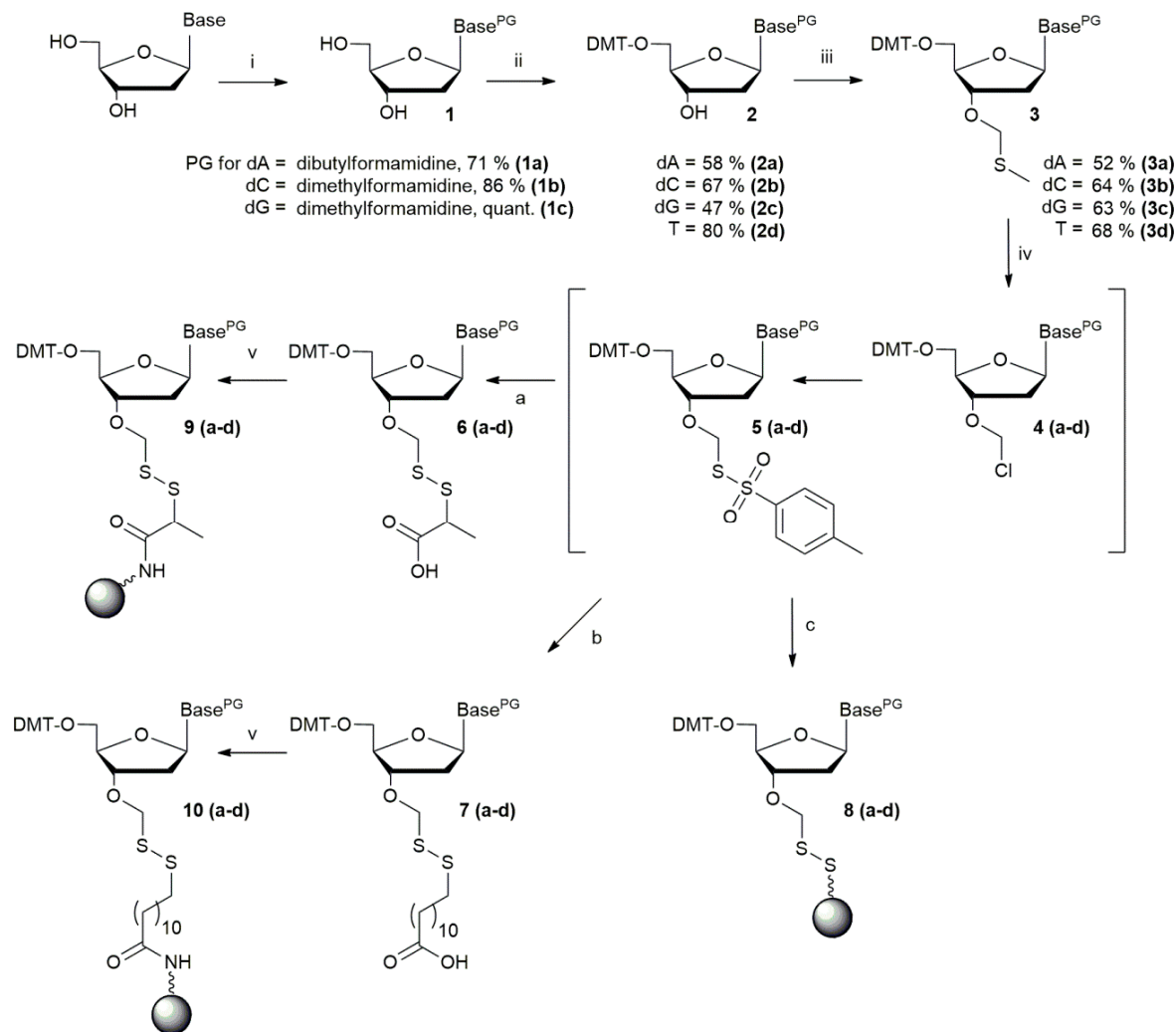
2.1. General Information

Chemicals were purchased from commercial suppliers (Roth, Sigma Aldrich, Merck, TCI). Organic solutions were concentrated by rotary evaporation at 40 °C. Pyridine was stored over KOH, distilled prior to use and stored over a molecular sieve. Buffers for the purification of trinucleotides were prepared with micro-pore water (Nanopore, Thermo Scientific). Products were visualized by thin layer chromatography (TLC) on aluminum sheets covered with silica gel 60 F254 (Merck) at 254 nm. Unless otherwise described, the standard TLC solvent system dichloromethane/methanol = 95:5 was used. Column chromatography was carried out on silica gel from Merck (Geduran Si 60, 0.063–0.200 mm). Solvents for column chromatography were purified by rotary evaporation. All buffers for HPLC analyses were filtered through a 0.2 µm acetonitrile resistant membrane and degassed in the ultrasonic bath for 15 min. MALDI-TOF analyses were done with a Bruker Daltonics Microflex in either positive or negative mode. For this, 1 µL sample solution was mixed with 1 µL hydroxypicolinic acid solution (in H₂O/acetonitrile = 1:1) on a plate (MSP 96 target ground steel) and dried to crystallization [11]. Determination of support loading densities was carried out by detritylation of the support-linked nucleoside and UV/VIS absorption measurements. Therefore, 3% dichloroacetic acid was added to the support. The absorption of the DMT-cation was measured at 498 nm, and the corresponding DMT concentration was determined by comparison to a standard curve. For the detailed procedure see Suchsland et al. [10] and Supplementary Materials S1.

List of solid supports used in this work: **A** = Amino CPG (controlled pored glass), 500 Ångström (105 µmol/g) from Chem Genes, (USA); **B** = Polystyrene A SH, 200–400 mesh (850 µmol/g) from RAPP Polymere (Germany); **C** = Polystyrene AM NH₂, 200–400 mesh (980 µmol/g, high loaded) from RAPP Polymere (Germany); **D** = Custom Primer Support Amino, high cross-linked polystyrene/divinylbenzene (200 µmol/g), from GE Healthcare (Sweden).

2.2. Synthesis of 5'-O-Dimethoxytrityl-3'-O-Methylthiomethyl-2'-Deoxynucleosides

The synthesis of 3'-O-thiomethyl-modified nucleosides (in Scheme 1(3a–d)) is described in detail in Suchsland et al. [10]. See Supplementary Materials S2 for ¹H and ¹³C NMR spectra.



Scheme 1. Reaction scheme of nucleoside functionalization and immobilization. Nucleotides are linked to the support via a disulfide bridge, with (a) short linker (sec. carbon), (b) long linker (prim. carbon), (c) direct connection; (i) dC/dG: 5 eq. dimethylformamide-dimethylacetal, 1 h, 50 °C, in dimethylformamide; dA: 1.4 eq. dibutylformamide-dimethylacetal, rt, overnight, 1 h, 80 °C, in dimethylformamide; (ii) 1.3 eq. 4,4'-dimethoxytrityl chloride, 3 h up to overnight in pyridine; (iii) 70 eq. dimethyl sulfoxide, 53 eq. acetic acid, 53 eq. acetic anhydride, rt, 20 h; (iv) 3 eq. triethylamine, 1 eq. sulfonyl chloride, 1.5 eq. potassium thiotosylate in dichloromethane. (a) 2 eq. 2-mercapto propionic acid, rt, 2 h; (b) 2 eq. 12-mercaptododecanoic acid, rt, 2 h; (c) solid support B, rt, 1 h; (v) 2 eq. TSTU, 5 eq. triethylamine, solid support (A/C/D), rt, overnight in dimethylformamide/dichloromethane/water.

2.3. Loading of Support

2.3.1. Thymidine as Starting Nucleoside

Loading of Polystyrene A SH (B)

5'-O-Dimethoxytrityl-3'-O-methylthiomethyl-thymidine (**3d**) (0.85 mmol) was coevaporated three times with dry dichloromethane and dissolved under dry conditions in dry dichloromethane (53.55 mmol, 3.42 mL). Triethylamine (2.55 mmol, 0.35 mL) was added and the solution stirred in an ice bath. Freshly, up to colorlessness, distilled sulfonyl chloride (0.85 mmol, 0.07 mL) was mixed with dichloromethane (10.2 mmol, 0.65 mL), and this solution was added slowly and dropwise to the reaction mixture, which turned orange. After 10 min in the ice bath, the solution was stirred for 5 min at room temperature. Potassium thiotosylate (1.28 mmol, 0.243 g) was mixed with dimethylformamide (10.2 mmol, 0.78 mL), and this solution was added to the reaction mixture dropwise, changing the color from orange to yellow. Formation of the intermediate product **5d** was observed by TLC. After stirring for 10 min, 820 mg solid support **B** (polystyrene A SH) was added, and the reaction mixture was stirred for 20 h at room temperature. Upon removal of the liquid, the support was washed with dichloromethane and methanol and dried in vacuo [12,13]. The loading density of the support **8d** was 238.43 $\mu\text{mol/g}$.

Loading of Amino-Functionalized Supports (A, C, D)

- Preparation of 2'-O-linker modified thymidine derivatives **6d** and **7d**

5'-O-Dimethoxytrityl-3'-O-methylthiomethyl-thymidine (**3d**) (2.32 mmol) was dissolved in dry dichloromethane (9.33 mL). Triethylamine (6.96 mmol, 0.96 mL) was added, and the reaction mixture was cooled in an ice bath. Freshly distilled sulfonyl chloride (2.32 mmol, 0.19 mL) in dichloromethane (1.77 mL) was added dropwise to the reaction mixture. The solution was stirred for 10 min in the ice bath. After warming up to room temperature, a solution of potassium thiotosylate (3.4 mmol, 0.65 g) in dry dimethylformamide (2.17 mL) was added dropwise to the reaction mixture. Formation of the intermediate product **5d** was observed by TLC. After stirring for 10 min 2-mercaptopropanoic acid (4.64 mmol, 0.38 mL) was added. The color of the reaction mixture turned to light red due to the loss of traces of the DMT protecting group. After 20 min the color of the solution changed to yellow. After stirring for 1.5 h the reaction mixture was washed with saturated aqueous sodium bicarbonate solution and brine. After drying over Na_2SO_4 and removing the solvent under vacuum, the residue (**6d**) was used for in situ coupling to solid support (**A**, **C**, and **D**) [12,13]. For synthesis of compound **7d**, mercaptododecanoic acid was used instead of 2-mercaptopropanoic acid. Synthesis was conducted in the same way as described above for 2-mercaptopropanoic acid, although at smaller scale (0.45 mmol of **3d** and 0.9 mmol of mercaptododecanoic acid).

- Loading of solid support: Amino CPG (**A**)

One eighth of the reaction mixture from above containing 5'-O-dimethoxytrityl-3'-O-(propanoic acid-2-dithiomethyl)-thymidine (**6d**) was dissolved in dimethylformamide (0.4 mL), dichloromethane (0.8 mL) and H_2O (0.1 mL). Triethylamine (2.3 mmol, 0.32 mL) and 2-succinimide-1,1,3,3-tetramethyluronium tetrafluoroborate (TSTU) (2.5 mmol, 0.72 g) were added. Afterward solid support **A** (60 mg, Amino CPG) was added, and the reaction mixture was stirred for 48 h at room temperature. The loaded solid support (**9d**) was washed with ethanol, dichloromethane, and water and dried in vacuo [14]. The loading was 10.5 $\mu\text{mol/g}$. The coupling of 5'-O-dimethoxytrityl-3'-O-(dodecanoic acid-12-dithiomethyl)-thymidine (**7d**) to Amino CPG was done under the same conditions. The loading of the resulting material, **10d**, was 7.9 $\mu\text{mol/g}$.

- Loading of solid support: Polystyrene AM NH₂ (C)

Loading of polystyrene support **C** with 2-mercaptopropanoic acid or 12-mercaptododecanoic acid was done as described for the loading of Amino CPG (**A**) with one sixth of the 5'-*O*-dimethoxytrityl-3'-*O*-(propanoic acid-2-dithiomethyl)-thymidine (**6d**) or 5'-*O*-di-methoxytrityl-3'-*O*-(dodecanoic acid-12-dithiomethyl)-thymidine (**7d**) reaction mixture and 145 mg Polystyrene AM NH₂ (C) (for loading densities see Table 1).

- Loading of solid support: Custom Primer Support Amino (D)

Loading of polystyrene support **D** with 2-mercaptopropanoic acid or 12-mercaptododecanoic acid was equally done with one third of the 5'-*O*-dimethoxytrityl-3'-*O*-(propanoic acid-2-dithiomethyl)-thymidine reaction mixture (**6d**) and 230 mg Custom Primer Support Amino (**D**) or with one sixth of the 5'-*O*-dimethoxytrityl-3'-*O*-(dodecanoic acid-12-dithiomethyl)-thymidine reaction mixture (**7d**) and 150 mg Custom Primer Support Amino (**D**) (for loading densities see Table 1).

Table 1. Loading densities of various supports and trinucleotide recovery upon reductive cleavage with TCEP.

Entry #	Solid Support	Linker	Starting Nucleoside	Loading [μg/mol]	Recovery of Trinucleotide
1	A Amino CPG	12-mercaptododecanoic acid	Thymidine	7.9	quantitative
2	A Amino CPG	2-mercaptopropanoic acid	Thymidine	10.5	quantitative
3	B Thiol PS	-	Thymidine	238.4	<10%
4	C Amino PS	12-mercaptododecanoic acid	Thymidine	245.0	<10%
5	C Amino PS	2-mercaptopropanoic acid	Thymidine	57.8	<10%
6	D Amino PS (hcl)	12-mercaptododecanoic acid	Thymidine	89.3	quantitative
7	D Amino PS (hcl)	2-mercaptopropanoic acid	Thymidine	58.9	quantitative
			Deoxyguanosine	51.6	quantitative
			Deoxycytidine	58.8	quantitative
			Deoxyadenosine	113.0	quantitative

CPG—controlled pored glass, PS—Polystyrene, hcl—highly crosslinked.

Capping of Unreacted Amino or Thiol Groups on Solid Supports A, B, C, and D

The unreacted free amino-groups on the solid supports **A**, **C**, and **D** as well as the free thiol-groups on solid support **B** were capped with capping reagents A and B (Cap A: 0.67 mL *N*-methylimidazole, 3.4 mL acetonitrile; Cap B: 0.84 mL acetic anhydride, 2.1 mL acetonitrile, 1.26 mL trimethylpyridine) while stirring for 15 min. After capping, the solid support was washed with acetonitrile and dried in vacuo.

2.3.2. Loading of 2'-Deoxyguanosine on Solid Support D

Starting with the 3'-*O*-methylthiomethyl functionalized guanosine derivative (**3c**) (0.6 mmol, 0.41 g) the synthesis of acid-modified nucleoside **6c** using 2-mercaptopropanoic acid (1.2 mmol, 0.13 mL) was carried out under the same condition as described for the thymidine derivative **6d** (see above). The resulting crude compound **6c** was used for in situ coupling to solid support. It was dissolved in dimethylformamide/dichloromethane = 1:4. TSTU (3 eq.) and triethylamine (5 eq.) were added to the reaction mixture. Afterward, 285 mg solid support **D** was added to the reaction mixture and stirred for 48 h. Upon removal of the liquid, the solid support was washed with ethanol, dichloromethane, and water, and dried in vacuo. The loading was 51.56 μmol/g.

2.3.3. Loading of 2'-Deoxyadenosine on Solid Support D

Starting with the 3'-*O*-methylthiomethyl functionalized adenosine derivative (**3a**) (0.12 mmol, 0.09 g) the synthesis of acid-modified nucleoside **6a** using 2-mercaptopropanoic acid (0.24 mmol, 0.02 mL) was carried out under the same condition as described for the thymidine derivative **6d** (see above). Half

of the resulting crude compound **6a** was used for in situ coupling to solid support. It was dissolved in dimethylformamide/dichloromethane/water = 5:1:1.25. TSTU (3 eq.) and triethylamine (5 eq.) were added to the reaction mixture. Afterward, 43 mg solid support **D** was added to the reaction mixture and stirred for 48 h. Upon removal of the liquid, the solid support was washed with ethanol, dichloromethane, and water, and dried in vacuo. The loading was 113 $\mu\text{mol/g}$.

2.3.4. Loading of 2'-Deoxycytidine on Solid Support D

Starting with 3'-O-methylthiomethyl functionalized cytidine derivative (**3b**) (0.28 mmol, 0.19 g) the synthesis of acid-modified nucleoside **6b** using 2-mercaptopropanoic acid (0.56 mmol, 0.047 mL) was carried out under the same condition as described for the thymidine derivative **6d** (see above). The resulting crude compound **6b** was used for in situ coupling to solid support. It was dissolved in dimethylformamide/dichloromethane = 1:4. TSTU (3 eq.) and triethylamine (5 eq.) were added to the reaction mixture. Afterwards, 136 mg solid support **D** was added to the reaction mixture and stirred for 48 h. Upon removal of the liquid, the solid support was washed with ethanol, dichloromethane, and water, and dried in vacuo. The loading was 58.82 $\mu\text{mol/g}$.

2.3.5. Capping of Unreacted Amino Groups on Solid Supports D

The free amino groups of the solid support were capped with capping reagents A and B (Cap A: 0.8 mL *N*-methylimidazole, 4.1 mL acetonitrile; Cap B: 1 mL acetic anhydride, 2.5 mL acetonitrile, 1.5 mL trimethylpyridine) while stirring for 30 min. After capping, the solid support was washed with acetonitrile and dried in vacuo.

2.4. Cleavage from Support

Choice of Cleavage Reagent

Two reagents, DTT and TCEP, were tested as reducing agents for the cleavage of the disulfide bridge linking the nucleotide to the support. To this end, loaded solid support **B** was incubated with either DTT or TCEP at 55 °C in 0.5 mL phosphate buffer (9.6 mM, pH 7) overnight. For results see Table 1. For details see Supplementary Materials S3.

2.5. Synthesis and Purification of Trinucleotides

Trinucleotides were synthesized by phosphoramidite chemistry in 1 μmol scale with β -cyanoethyl- or methyl-protected standard phosphoramidites from LGC Link, Scotland and benzylmercaptotetrazole as activator [10]. Syntheses were carried out with a Gene Assembler Special from Amersham Biosciences, with a coupling time of 1.5 min and in DMT-on mode.

2.5.1. Trinucleotide Cleavage from Solid Support

Trinucleotides were cleaved from support with 300 μmol TCEP in 600 μL acetonitrile and 400 μL HEPES-buffer (100 mM, pH 7) at 55 °C in an ultrasonic bath overnight. Afterward the solid supports were treated with 3% dichloroacetic acid to check if cleavage of the trinucleotide was completed. Eventually the remaining trinucleotides would be detected by the red color of the cleaved-off DMT cation.

2.5.2. Precipitation of Trinucleotides

Upon cleavage, the solid support was washed with water, acetonitrile, ethanol, acetone, tetrahydrofurane, and ethylacetate. Washing solutions were combined and, after evaporation, fully protected trinucleotides were precipitated from water.

3. Results and Discussion

Due to the obvious advantages of solid phase synthesis over solution chemistry, we sought to develop a strategy that allows the assembly of fully protected trinucleotides on solid support. The key issue is the detachment of the trinucleotide from the support maintaining all protecting groups. Reductive cleavage is a suitable strategy, as it is orthogonal to the acid labile DMT group, as well as to nucleophilic removal of the phosphate and nucleobase protecting groups (Figure 1) [15–17]. Thus, we took an example from a previously published strategy for the synthesis of RNA using 2'-*O*-*tert*-butyldithiomethyl-protected building blocks [18], and developed a protocol for trinucleotide assembly with the starting nucleoside connected to the solid support by a dithiomethyl linkage (Figure 1). Reductive cleavage of the disulfide bond would produce a *S,O*-hemiacetal, which spontaneously decomposes into alcohol and thioformaldehyde, thus yielding the free 3'-OH group [18], which subsequently can be phosphitylated as described in our earlier work on the preparation of trinucleotide synthons [6].

For immobilization of the starting nucleoside, first the 3'-OH group needed to be appropriately functionalized (Scheme 1). This was achieved by the site-specific introduction of a methylthiomethyl group, followed by activation of the obtained 3'-*O*-methylthiomethyl (3'-*O*-MTM) derivatives **3a–d** by treatment with sulfuryl chloride to produce the 3'-*O*-chloromethyl ether (**4a–d**) in a Pummerer rearrangement [19,20]. The chloromethylether reacts with potassium thiotosylate forming the reactive compound **5a–d**, which subsequently is linked to the polymer via a disulfide bridge [12,13]. 5'-*O*-Dimethoxytritylated nucleosides were used as starting compounds requiring careful control of reaction conditions (pH), in particular during the first step of MTM introduction.

3'-*O*-MTM derivatives **3a–d** of all four deoxynucleotides were prepared with satisfying yields (52%–68% [10]) and linked either directly to thiol-derivatized polystyrene **B** (**8a–d**) or, via a bifunctionalized linker, to amino-derivatized polystyrene **C** and **D** (**10a–d**) or amino-derivatized CPG **A** (**9a–d**). As a bifunctionalized linker, we tested 2-mercaptopropionic acid or, as a longer variant, 12-mercaptodecanoic acid. Both linker molecules comprise a thiol group for disulfide formation, however in the shorter variant it is connected to a secondary carbon and in the longer variant it is connected to a primary carbon atom. Thus, the formation of the disulfide bridge, its stability during trinucleotide assembly, and disulfide cleavage can be studied depending on linker lengths and type of the thiol. After attachment of the linker to nucleoside derivatives (**6a–d**, **7a–d**), immobilization to aminopolystyrene or Amino CPG was achieved by amide bond formation upon activation of the linker carboxyl group with TSTU [14].

Seven types of support, each consisting of either polystyrene or CPG, linked directly or via a bifunctionalized linker to thymidine as the starting nucleoside, were prepared for initial testing (Figure 2, Table 1). The loading was determined by removal of the DMT group under acidic conditions according to Tang et al. [11] (for the detailed procedure see Suchsland et al. [10]). In general, the loading of polystyrene (**B**, **C**, **D**) was found to be higher (58–245 $\mu\text{mol/g}$) than that of Amino CPG **A** (8–11 $\mu\text{mol/g}$). Removal of the nucleoside from the support was tested by treatment with either a buffered solution of 1,4-dithiothreitol (DTT, pH7) at 50 °C or tris-(2-carboxyethyl)-phosphine (TCEP, pH 7) at 55 °C in the ultrasonic bath for 20 h. The same assay as used above for the determination of support loading with nucleoside was applied for analyzing nucleoside/trinucleotide recovery. To this end, upon reductive cleavage of the disulfide bridge linking the starting nucleoside to the support, and extensive washing, the support was treated with 3% dichloroacetic acid, and the absorption at 498 nm was measured to detect any remaining nucleoside by detritylation. Interestingly, there was always DMT detectable when polystyrene **B** and **C** were treated with 3% dichloroacetic acid after disulfide cleavage (Table 1, entry #3, #4, and #5). This may be the result of either inefficient cleavage of the disulfide bridge, such that trinucleotides remained covalently linked to the polymer, or, trinucleotides upon disulfide cleavage non-specifically interact with the polystyrene, such that they cannot be washed off completely. This was different for CPG and highly crosslinked (hcl) polystyrene (Table 1, entries #1, #2, #6, and #7), which are the typical supports used in oligonucleotide synthesis, and thus, not surprisingly, showed best results.

TCEP was found being more efficient (see Supplementary Materials S3) [21] and, due to the intended use of methyl-protected phosphoramidites (see below), it was the better choice for the cleavage of the synthesized trinucleotides from the support. Overall, highly crosslinked polystyrene showed the best features in terms of nucleoside loading and TCEP-mediated detachment, independent of the linker connecting the starting nucleoside to the amino-functionalized polymer. Thus, hcl polystyrene would be the best choice for trinucleotide assembly, in particular, also when the process needs to be carried out at a larger scale.

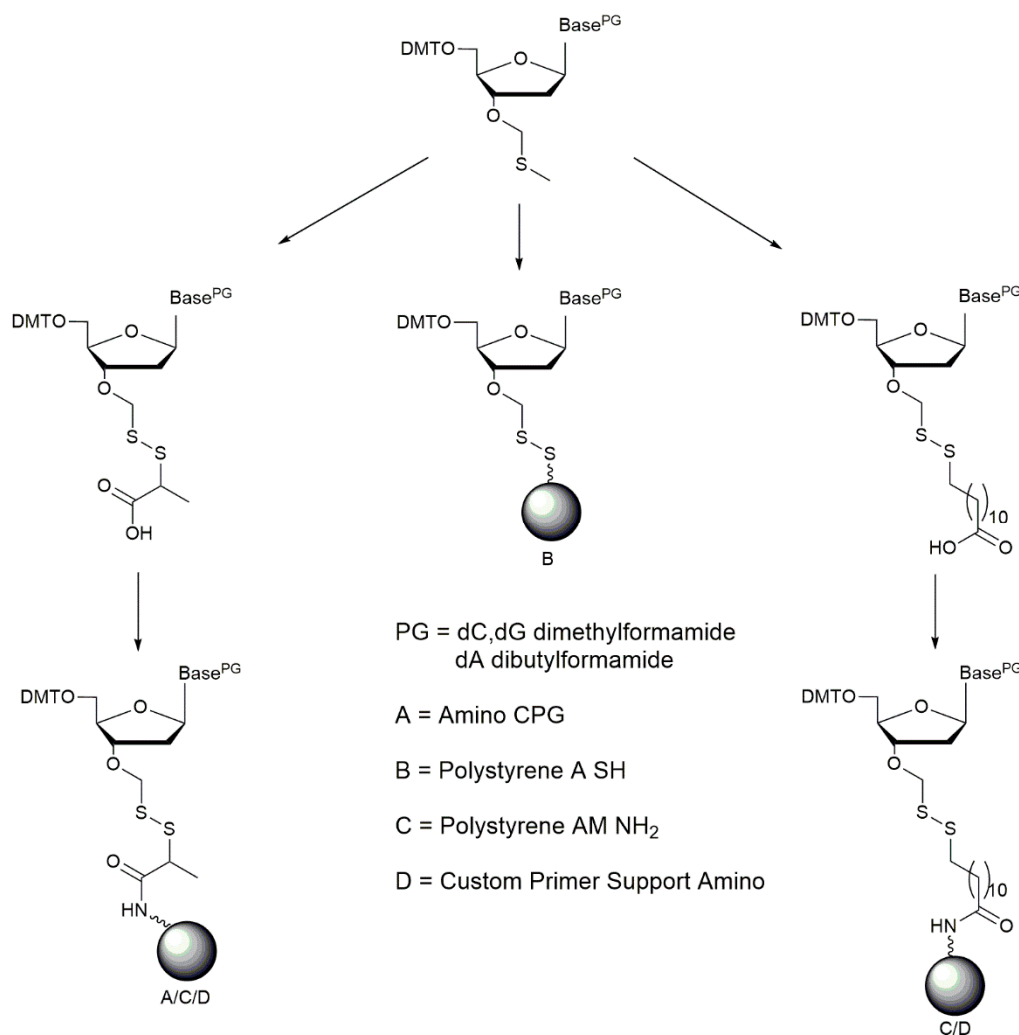


Figure 2. Overview of supports loaded with starting nucleoside.

In previous experiments we had investigated the influence of the nature of the linker thiol being primary, secondary, or tertiary. The stability of a disulfide bridge depends on the nature of the bridge-forming thiol, with a tertiary thiol being most stabilizing due to the electron-donating effect of the tertiary alkyl group. In our preliminary experiments, we found the disulfide bridge formed with the primary thiol was clearly more labile than those formed with the secondary and tertiary thiols, when it was cleaved with DTT (see Supplementary Materials S4 for details). Under the conditions of disulfide cleavage used here (TCEP, over 20 h), this stability difference could not be observed, since cleavage from support A and D, with either 12-mercaptododecanoic acid or 2-mercaptopropanoic acid as linker was completed after a rather long incubation time. Nevertheless, based on our previous results with DTT, we decided to use 2-mercaptopropanoic acid as linker for our further studies, because of the higher stability of the formed disulfide bridge and, hence, less risky trinucleotide assembly. Thus, in addition to T, the 5'-O-DMT-3'-O-MTM-functionalized derivatives of dA, dC, and dG (**3a–c**, Scheme 1), were also

linked to 2-mercaptopropionic acid followed by immobilization on amino-functionalized polystyrene (D) (Table 1).

Before use of the loaded supports, free amino groups were capped by treatment with acetic anhydride. Then, trinucleotides were synthesized by coupling of either β -cyanoethyl- or methyl-protected phosphoramidites of *N*-acyl-5'-O-DMT-protected 2'-deoxynucleosides. Previously, we had used β -cyanoethyl-protected phosphoramidites for trinucleotide assembly in solution and found that the β -cyanoethyl group was suitable for phosphate protection. It was stable throughout trinucleotide assembly and phosphitylation, provided that particular attention was paid to reaction and purification conditions, in particular to the pH of reaction media, solvents, eluents, and buffers. In order to circumvent these challenges and to allow easier handling of the fully protected trinucleotides during purification and in further reactions, we later switched to the more stable methyl group for phosphate protection. Altogether, ten different trinucleotides were synthesized basically following the standard protocol for DNA synthesis [10]. For initial testing, syntheses were carried out at 1 μ mol scale. Three trimeric oligodeoxyribonucleotides were assembled from β -cyanoethyl phosphoramidites, and seven were assembled from methyl phosphoramidites (Table 2). For oxidation of the phosphite, a diluted solution of iodine (0.02 M) was used, because of the previously observed partial cleavage of the disulfide bond when treated with 0.1 M iodine solution [18,22].

Table 2. Overview of synthesized trinucleotides.

Phosphate Protecting Group	Trinucleotide	Mass Calculated	Mass Found	Cleavage Reagent
Me	ATT	1260.14	1284.84 (+Na)	TCEP
Me	AAT	1339.24	1385.82 (+2Na)	TCEP
Me	TTT	1180.34	1180.30	TCEP
Me	AAA	1402.50	1448.22 (+2Na)	TCEP
Me	GGA	1449.49	1472.49 (+Na)	DTT
Me	GGC	1459.47	1482.34 (+Na)	DTT
Me	GGT	1370.44	1393.38 (+Na)	DTT
β -cyanoethyl	TTT	1258.37	1281.29 (+Na)	DTT
β -cyanoethyl	TTA	1337.42	1360.34 (+Na)	DTT
β -cyanoethyl	TTC	1347.39	1370.38 (+Na)	DTT

Upon cleavage from the support with a buffered solution of DTT, the quality of fully protected trinucleotides TTT, TTA, and TTC carrying the β -cyanoethyl group at the phosphates was confirmed by HPLC and MALDI-TOF MS (Table 2 and Supplementary Materials S5). However, when using methyl-protected phosphoramidites, partial loss of the methyl group was observed upon removal of trinucleotides from the support with DTT, due to nucleophilic attack of the DTT-SH group onto the methyl group carbon (Supplementary Materials S6). On the contrary, when trinucleotides were removed by disulfide cleavage with TCEP under the conditions mentioned above, the methyl protection at the phosphates remained intact (Supplementary Materials S7). Upon cleavage from the support, fully protected trinucleotides were precipitated from water, dried, and stored under vacuum.

Trinucleotides should be phosphitylated directly prior to use in DNA synthesis. Particular care has to be taken when β -cyanoethyl-protected trinucleotides are phosphitylated, as we have described recently [6]. Here, the lability of the β -cyanoethyl group under basic conditions requires careful choice of the phosphitylation reagent and control of reaction conditions. The same optimized conditions can be applied to methyl-protected phosphoramidites, although the methyl group at the phosphorous is less labile.

4. Conclusions

In conclusion, assembly of trinucleotides on solid support with the starting nucleoside being linked to the polymer via a disulfide bridge is a straightforward strategy for the preparation of

fully protected trimeric oligodeoxyribonucleotides. Upon cleavage of the disulfide under reducing conditions, the trimer with all protecting groups at the nucleobases, the phosphates, and the terminal 5'-OH-group remaining intact is released into solution. Following evaporation of the solvent, trimers are precipitated from water, dried, and stored until use in standard DNA synthesis, starting with phosphitylation as described [6]. The solid phase strategy nicely adds to our previously developed approach for the preparation of trinucleotide synthons in solution and to the more recently published methods for trinucleotide assembly on a precipitative soluble support [8,23]. So far, syntheses were carried out at 1 μ mol scale to demonstrate the suitability of the method. In future work, fully protected trimers will be prepared at a larger scale to produce sufficient amounts for further evaluation of the trimers in gene library synthesis.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2076-3417/9/11/2199/s1>, Figure S1. Standard curve of DMT-Cl for calculation of loading density, Figure S2. Synthesis of 5'-O-dimethoxytrityl-3'-O-dithiomethyl-N-benzoyl-2'-deoxycytidine with primary, secondary, or tertiary disulfide bridge (S-prim/sec/tert), Figure S3. Synthesized trinucleotides with primary, secondary, and tertiary disulfide bridge ATC-S_{prim}, CAC-S_{sec}, TTC-S_{tert} (* calculated mass), Figure S4. Reductive cleavage of the 3'-disulfide bridge of trinucleotides ATC-S_{prim}, CAC-S_{sec}, and TTC-S_{tert}. Shown are the mass spectra of trinucleotides after 30 min and 9 h of treatment with DTT, Figure S5. MS-Spectra of trinucleotides after cleavage from the support, Figure S6. RP-HPLC of TTT-3'-OH after cleavage from solid support, buffer A: 5% acetonitrile, buffer B: 70% acetonitrile, VP 125/21 Nucleosil 120-10 C18, Figure S7. RP-HPLC TTA-3'-OH after cleavage from solid support, buffer A: 5% acetonitrile, buffer B: 70% acetonitrile, VP 125/21 Nucleosil 120-10 C18, Figure S8. RP-HPLC TTC-3'-OH after cleavage from solid support, buffer A: 5% acetonitrile, buffer B: 70% acetonitrile, VP 125/21 Nucleosil 120-10 C18, Figure S9. MS-Spectra of trinucleotides with methyl/protected phosphate after cleavage with DTT from the support. The difference of 14 indicates loss of a methyl group at the phosphate, Figure S10. MS-Spectra of trinucleotides with methyl-protected phosphate after cleavage with TCEP from the support, Table S1. Cleavage conditions, Table S2. 3'-OMT-S-prim/sec/tert yields and masses.

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